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REVIEW

Multispecies communities: interspecies interactions influence growth on saliva as sole nutritional source

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Human oral bacteria live in multispecies communities in the biofilm called dental plaque. This review focuses on the interactions of seven species and the ability of each species individually and together with other species to grow on saliva as the sole source of nutrient. Community formation in biofilms in flow cells is monitored using species-specific fluorophore-conjugated immunoglobulin G, and images are captured by confocal microscopy. Early colonizing veillonellae emerge from this review of interspecies interactions in saliva as a critical genus that guides the development of multispecies communities. Highly selective interspecies recognition is evident as initial colonizers pair with early and middle colonizers to form multispecies communities that grow on saliva.

***Keywords*:** growth on saliva; multispecies communities; biofilm; flow cell; interspecies interactions

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Introduction

The microbial communities that form on freshly cleaned tooth surfaces are composed of multiple species that exist as a network of cell-to-cell interspecies interactions. Even at the earliest times of initial colonization, flowing saliva bathes both cleaned surfaces and already attached cells with a variety of species suspended in saliva. A highly selective mechanism of coaggregation between species is involved in the development of multispecies communities [1-3]. Many of these interspecies and intra-species coaggregations are reversible by the addition of simple sugars such as lactose. The primary initial colonizers are streptococci and some actinomyces, and early colonizing veillonellae coaggregate with streptococci and actinomyces. Not surprisingly, a micromanipulated initial community comprised of two streptococcal species and a *Veillonella* sp. [4] coaggregated with each other, and some of the coaggregations were lactose-reversible. Lactose-reversible coaggregation was previously shown to be a factor in the *Actinomyces-Streptococcus* initial communities [5]. Accompanying the physical coaggregation reactions are the interspecies metabolic interactions, the small-molecule chemical signaling among species, and cellular growth. All of these properties of biofilm development are significant and contribute to dental plaque accumulation. The emphasis of this review is on the outcome of these interspecies interactions: growth on saliva as a sole nutrient!

Cellular growth on saliva as sole nutritional source

Early studies by van der Hoeven and colleagues [6-7] on the ability of oral bacteria to grow on saliva as the sole source of nutrient showed that an *Actinomyces* species could grow, but three *Streptococcus* species could not grow on saliva. A wide variety of cell-associated hydrolytic enzymatic activities, including glycosidases, peptidases and esterases were measured and led to the proposal that specific binding by oral bacteria to salivary glycoproteins is a possible mechanism to localize nutrients in proximity to the cell [6]. These high molecular-weight glycoproteins, known as mucins, are composed of numerous oligosaccharide side-chains O-glycosidically linked

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to the protein core and serve as a nutritional source for microbial growth.

Hog gastric mucin has been used as a model substrate for saliva because this mucin possesses the highest similarity in oligosaccharide structure with human salivary mucin [8]. The idea that different bacterial species possess individual enzymatic activities that act in succession was explore in experiments using hog gastric mucin as the sole source of carbohydrate for growth and synergistic degradation of mucin was observed by van der Hoeven and Camp [9]. These investigators reported that *Streptococcus sanguis* (*S. sanguis*) Ny 584 reached significantly higher cell densities in mixed chemostat cultures with *Streptococcus oralis* (*S. oralis*) Ny 586 than in pure culture [9], supporting the hypothesis that complementary enzymatic activities from each species coordinate their actions to increase the efficient use of this mucin.

Marsh and co-workers [10] expanded this type of investigation by using chemostats as growth chambers, hog gastric mucin as the main nutritional source, and ten-species communities. The species contributed complementary hydrolytic enzymatic activities to the community, thus indicating metabolic cooperation. These studies were broadened to include the influence of anaerobic environments and role of coaggregation among the ten species in establishing a stable multispecies community [11]. These investigators showed that coaggregation between *Fusobacterium nucleatum* (*F. nucleatum*) and other species, in particular black-pigmented anaerobes such as *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella nigrescens* (*P. nigrescens*), facilitated the survival of these obligate anaerobes in oxygenated environments.

Wickstrom and colleagues [12-13] explored the proteolytic degradation of a single but complex human salivary oligomeric glycoprotein mucin MUC5B, as a sole nutritional source for oral bacteria. Dental plaque degraded the mucin polypeptide backbone; a 4-species consortium also degraded MUC5B, although individually, each species could not [12]. Collectively, these investigations demonstrated that, when the species within oral multispecies communities cooperated, the communities grew rapidly on salivary and related mucins.

Biofilm growth on saliva in flow cells

With these studies as a backdrop, my laboratory initiated a series of experiments using an anaerobic flow cell system, fluorescently labeled antibodies, and confocal laser scanning microscopy to capture images of biofilm growth. Filter-sterilized saliva [6] was 1∶4 diluted with sterile water and used as the sole nutritional source. Our flow cell system was based on the parallel plate flow cell system designed and characterized by Busscher and colleagues [14-16], who showed its versatility in studies with coaggregating oral streptococci and actinomyces [17]. Palmer *et al*. [18] reported that *Streptococcus gordonii* (*S. gordonii*) DL1 could grow on saliva, but *S. oralis* 34 and *Actinomyces oris* (*A. oris*) T14V could not; however, the latter two species together showed luxuriant growth, indicating a mutualistic relationship. Egland *et al*. [19] showed the critical significance of juxtaposition of streptococci and veillonellae in cell-cell signaling in a flow system, but juxtaposition in a static system was not important. As a mimic of naturally flowing saliva bathing oral surfaces, the flow cell model became a standard tool for investigations of multispecies community growth on saliva as a sole nutritional source.

The first objective was to determine the range of single-species that are able to grow on saliva in a flowing environment. As some of the earlier researchers reported, a few species can grow but most cannot grow individually on saliva. By pairing species, many that were unable to grow individually showed mutualistic growth in certain pairings but not in others. For example, *Veillonella parvula* (*V. parvula*) PK1910, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) JP2, and *F. nucleatum* ATCC 10953 cannot grow on saliva as single-species biofilms [20]. It should be noted here that *V. parvula* PK1910 is the same as *Veillonella atypica* (*V. atypica*) PK1910 [21] and *Veillonella* sp. strain PK1910 [22]. This strain was originally classified as *V. atypica* on the basis of physiological traits, but after conducting phylogenetic characterization of 16S ribosomal RNA gene sequences, it was found to be closely related to *V. parvula* strains [23] and has been confirmed by Chalmers, *et al*. [4] and Qi and Ferretti [24]. Although PK1910, JP2, and ATCC 10953 cannot grow individually, pairwise each grows mutualistically, and they grow as a three-species community (Figure 1) [20].

Also commonly observed is a three-species community that does not grow on saliva, although pairwise the species grow well together. For example, as a two-species community *A. oris* ATCC 43146 and *P. gingivalis* ATCC 33277 grow 2-fold and 12-fold, respectively over an 18 hour period [25]. *V. parvula* PK1910 and *A. oris* ATCC 43146 exhibit 7-fold increases each, *V. parvula* PK1910 and *P. gingivalis* ATCC 33277 each exhibit a 5-fold increase when paired; however, the three-species community does not grow. Or, the reverse outcome can occur, for example, *S. oralis* 34 and *P. gingivalis* ATCC 33277 do not grow when paired, but addition of *V. parvula* PK1910 promotes 9-fold, 9-fold, and 13-fold, respectively,

increases in cell volumes [22]. Thus, the early colonizer *P. gingivalis* does not pair with the initial colonizer *S. oralis*, but addition of another early colonizer *V. parvula* elicits nearly 10-fold growth in just 18 hours incubation in a flow cell. However, *V. parvula* was not able to promote growth in all three-species combinations, as evident in the *A. oris*-*P. gingivalis*-*V. parvula* grouping described above. These results illustrate the high species specificity for productive multispecies community growth on saliva and support the concept of sequential colonization of enamel surfaces.

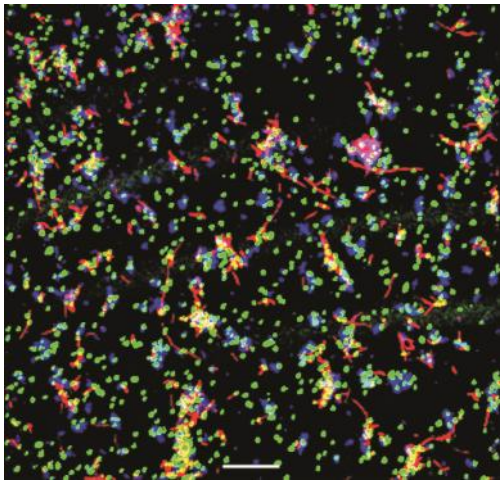

Figure 1 Representative confocal micrograph of mutualistic human oral bacteria, *Veillonella parvula* PK1910 (blue) with *A. actinomycetemcomitans* JP2 (green) and *F. nucleatum* ATCC 10953 (red) showing integrated multispecies biofilm communities after 18 h in a flow cell fed solely with saliva. Slender rod-shaped fusobacteria are always in contact with smaller spherical veillonellae or short-rod aggregatibacteria, and some interspecies interactions have the appearance of "corncobs", which are common in natural dental plaque. Bacterial cells were stained with species-specific fluorophore-conjugated immunoglobulin G, and cell-to-cell contact is evident. Bar marker is 50 μm.

The addition of *A. actinomycetemcomitans* JP2 to the pair *S. oralis* 34 and *P. gingivalis* ATCC 33277 yields no growth of any species, although the pairing of *A. actinomycetemcomitans* JP2 and *P. gingivalis* ATCC 33277 yields 4-fold increases of each species. Likewise, the pairing of *F. nucleatum* ATCC 10953 with *P. gingivalis* ATCC 33277 yields 9-fold and 3-fold, respectively, increases in biovolume, but addition of *S. oralis* 34 results in growth of none of the three species [25]. Thus, *S. oralis* appears to be highly specific for *V. parvula* and likely for other veillonellae. Streptococci produce significant amounts of lactic acid, and veillonellae utilize lactic acid as their source of carbon and energy. This metabolic connection has been discussed and expanded [26], and it might be a driving force in the development of multispecies communities and in the transition of initial colonizers as promoters of early and middle colonizations.

Lastly, the highest productive growth on saliva by a three-species community was accomplished by the community composed of *S. oralis* 34, *V. parvula* PK1910, and *F. nucleatum* ATCC 10953, which exhibited increases of 163-fold, 100-fold, and 23-fold, respectively, in 18 hours of incubation [22]. However, only marginal growth of *S. oralis* 34 was observed after replacing *V. parvula* PK1910 with *A. oris* ATCC 43146 in a three-species community with *F. nucleatum* ATCC 10953 (Figure 2) [27]. Thus, although *S. oralis* plus *A. oris* grow mutualistically [18], and *S. oralis* plus *V. parvula* grow mutualistically [22], their respective partnerships with *F. nucleatum* lead to very different outcomes.

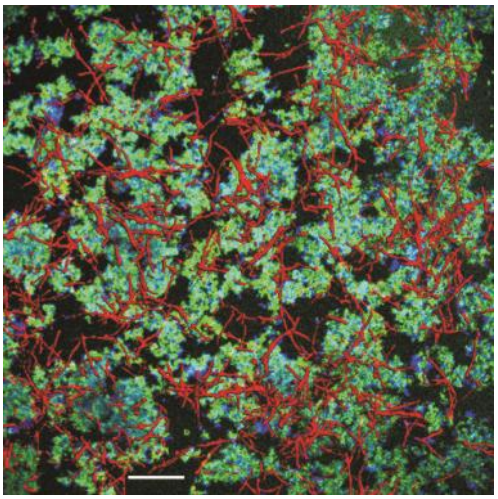

Figure 2 Representative confocal micrograph of a three-genera biofilm grown in a flow cell using saliva as the sole nutritional source for growth. The multispecies communities at 18-hour show intimate interactions of *S. oralis* 34 (blue) with *A. oris* ATCC 43146 (green) and *Fusobacterium nucleatum* ATCC 10953 (red). Bacterial cells were stained with species-specific fluorophore-conjugated immunoglobulin G, and cell-to-cell contact is evident. Bar marker is 50 μm.

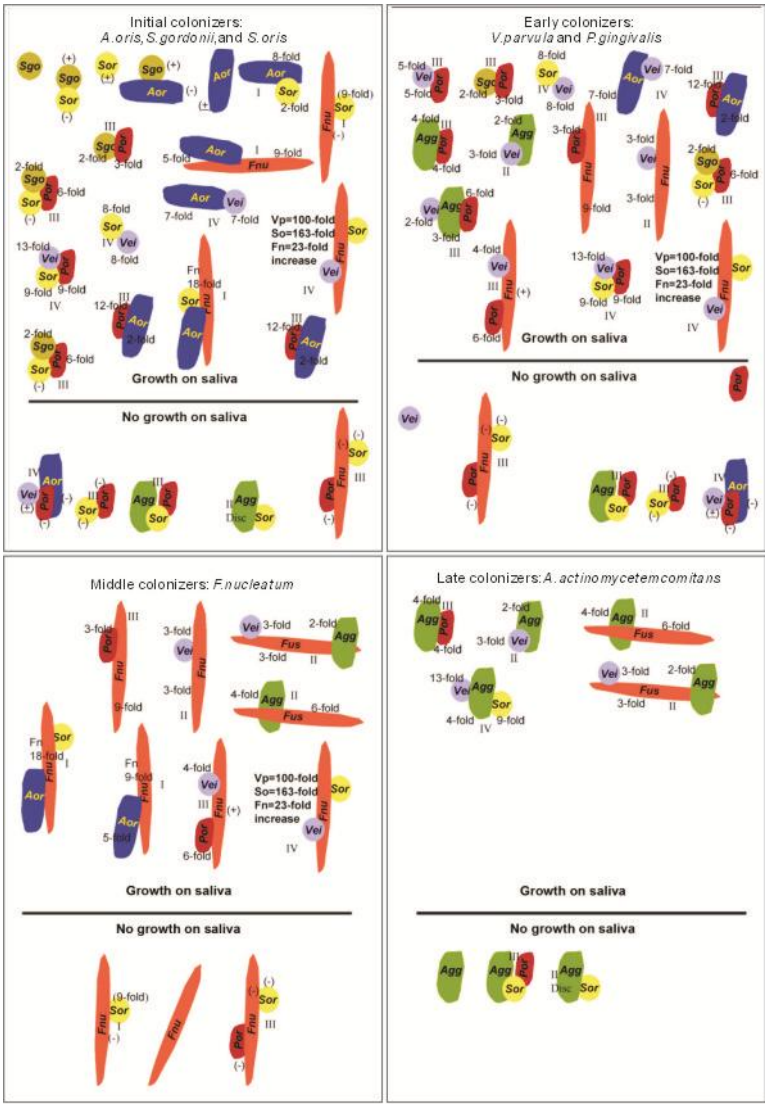

Figure 3 Growth on saliva as the sole source of nutrition by individual species, species pairs, and three-species communities, all of which are depicted as different cell shapes and colors. Individual species, species pairs and three-species communities that grow on saliva appear above the horizontal line in each of the four panels; those that do not grow on saliva appear below the line. Upper left panel: initial colonizers *A. oris*, *S. gordonii*, and *S. oralis* and their pairs and three-species communities with early, middle and late colonizers. Upper right panel: early colonizers *P. gingivalis* and *V. parvula* and their pairs and three-species communities with initial, middle and late colonizers. Lower right panel: middle colonizer *F. nucleatum* and its pairs and three-species communities with initial, early and late colonizers. Lower left panel: late colonizer *A. actinomycetemcomitans* and its pairs and three-species communities with initial, early and middle colonizers.

The amount of growth of each species between 4 and 18 hours of incubation in the flow cell is given as "fold" increases; for example, the *V. parvula* (Vei) + *P. gingivalis* (Por) pair shown in the early colonizers (upper right panel; top left pair) indicates that each species increased 5-fold. Species that grew less than 2-fold are indicated as (+); species that did not grow in pairings or three-species communities are indicated as (-); a few combinations showed growth sometimes but not other times, and this is indicated as (+). The roman numerals next to the species groupings refer to four publications: I= [27]; II= [20]; III= [25]; IV= [22].

Aor: *A. oris* ATCC 43146; Agg: *A. actinomycetemcomitans* JP2; Fus: *F. nucleatum* ATCC 10593 (Fn); Por: *P. gingivalis* ATCC 33277; Sgo: *S. gordonii* DL1; Sor: *S. oralis* 34 (So); Vei: *V. parvula* PK1910 (Vp).

Central role of veillonellae and role of other species in sequential colonization of enamel surfaces

The interspecies interactions discussed in the above section illustrate possible routes of influence for each species in the development of dental plaque biofilms. The above interactions and numerous others are summarized pictorially in Figure 3. Initial colonizers, such as *S. oralis*, *S. gordonii*, and *A. oris* appear to interact productively with early colonizers, such as *V. parvula* and *P. gingivalis* (Figure 3). In sharp contrast, these initial colonizers do not grow pairwise with each other, with the exception of *S. oralis* with *A. oris*. Early colonizers appear to have a broader interactive range that includes pairwise matches with initial, middle and late colonizers.

Middle colonizer *F. nucleatum* also exhibits a broad range of pairwise interactions, and its three-species community with *V. parvula* and *S. oralis* is the most productive of any so far examined (Figure 3). This community appears to benefit exceptionally well from the cross-feeding associated with streptococcal production of lactic acid and veillonellae consumption of lactic acid. Late colonizer *A. actinomycetemcomitans* interacts well with many species belonging to earlier colonization steps, which supports the idea of sequential colonization.

One point that should be emphasized here is the contribution of *V. parvula* PK1910 to multispecies community growth (Figure 3, early colonizers panel). *V. parvula* PK1910 has a positive influence on the success of communities to grow, although by itself it is unable to grow on saliva. This property of enhancing community growth with a variety of partners is appropriate for an early colonizer, as it coordinates a succession of species that colonize the tooth surface. In contrast, *S. oralis* 34 appears to be much more restricted in its successful partnerships, since many of the pairs and three-species groups are unable to grow on saliva (Figure 3). However, the evidence presented in Figure 3 shows the distinctive partnership of *S. oralis* and *V. parvula*, either pairwise or in nourishing three-species communities with *P. gingivalis* (9-, 13-, 9-fold, respectively, increases in growth), or *F. nucleatum* (163-, 100-, 23-fold, respectively, increases in growth), or *A. actinomycetemcomitans* (9-, 13-, 4-fold, respectively, increases in growth). Collectively, the results show the critical significance of using particular combinations of species for studies of multispecies community growth on saliva.

Summary and future direction

Early concerns about using saliva as a sole nutritional source included the concern that results obtained with such an undefined nutrient might be unrepeatable and possibly unreliable. However, this turned out to be unfounded. All of the results in Figure 3 have been repeated several times; some were repeated with many different batches of saliva over a period of several years.

The results discussed in this review strongly support a role for interspecies interactions in the repetitive and sequential colonization of oral bacterial species in dental plaque formation. Cell-to-cell interactions and metabolic exchanges among species appear to be key elements in successful, productive multispecies communities. A prime example of interspecies interactions is the *S. oralis*-*V. parvula* pair, which promote growth of three-species communities.

The involvement of mutualistic relationships in three-species communities offers opportunities to uncover the mechanisms that lead to mutualism. More genetic tools are needed to aid in this discovery. Exposing whole-community gene expression will greatly expand our knowledge of interspecies interactions in multispecies communities.

The saliva we used for these studies was prepared by sterile filtration [6], but a recent method involving gamma irradiation preserves enzyme activity and maintains the integrity of salivary proteins and mucins [28]. Unlike sterile filtration that could remove about one half of the total protein, gamma irradiation does not, but the latter promising method has yet to be tested as a treatment procedure to produce a nutritional source for microbial growth. The intent of this review is to stimulate research in the use of the natural flowing nutritional source for oral bacteria: saliva.

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